

*Commentary***Title:** Poly(2-oxazoline) Hydrogels as Next Generation Three Dimensional Cell Supports

Tim R. Dargaville<sup>1\*</sup>, Brett G. Hollier<sup>2</sup>, Ali Shokoohmand<sup>2</sup>, Richard Hoogenboom<sup>3\*</sup>

1. Cells and Tissue Domain, Queensland University of Technology, Institute of Health and Biomedical Innovation, 60 Musk Avenue, Kelvin Grove, 4059 Queensland, Australia

2. Cells and Tissue Domain, Queensland University of Technology, Institute of Health and Biomedical Innovation, Translational Research Institute, 37 Kent St, Woolloongabba, 4102 Queensland, Australia

3. Supramolecular Chemistry Group, Department of Organic Chemistry, Ghent University, Krijgslaan 281 S4, B-9000 Ghent, Belgium

Corresponding authors: [t.dargaville@qut.edu.au](mailto:t.dargaville@qut.edu.au), [Richard.Hoogenboom@UGent.be](mailto:Richard.Hoogenboom@UGent.be)

*Abstract*

Synthetic hydrogels selectively decorated with cell adhesion motifs are rapidly emerging as promising substrates for 3D cell culture. When cells are grown in 3D they experience potentially more physiologically relevant cell-cell interactions and physical cues compared with traditional 2D cell culture on stiff surfaces. A newly developed polymer based on poly(2-oxazoline)s has been **used for the first time** to control attachment of fibroblast cells and is discussed here for its potential use in 3D cell culture with particular focus on cancer cells towards the ultimate aim of high throughput screening of anti-cancer therapies. Advantages and limitations of using poly(2-oxazoline) hydrogels are discussed and compared with more established polymers, **especially polyethylene glycol (PEG)**.

*Introduction:*

In recent years, scientist have prospected great impact of three-dimensional (3D) cell culture, including a *revolution in science and medicine* enabled by accurate reproduction of healthy or diseased tissue *in vitro*.<sup>1</sup> To realise this lofty and paradigm shifting goal an overhaul of cell culture technology is needed and, to do so, new materials must be developed to replace the ubiquitous flat tissue culture flask. The interest in 3D cell culture is not surprising given the dissimilarities between the stiff plastic used in the majority of culture vessels and the soft extracellular matrix in which cells naturally grow.<sup>2</sup> Cancer researchers were amongst the first to adopt 3D soft matrices as the substrate of choice for culturing cancer cells where multi-cellular spheroids can mimic the organization observed in tumors<sup>2</sup> These spheroids have proven extremely useful in researching anti-proliferative drugs and cancer cell biology and offer more relevant information compared with 2D cultures, which poorly predict the chemosensitivity of drugs.

There are numerous strategies available for growing cells in 3D scaffolds based on natural or synthetic materials with forms ranging from rigid porous architectures to soft hydrogels. For hard tissue, such as bone, 3D printing of thermoplastics has proven successful,<sup>3</sup> but for most other tissue types soft hydrogel scaffolds, that mimic the biomechanical properties of the ECM, are more appropriate and are the focus of this commentary. The most common methods for creating 3D hydrogels for culture of cancer cells involves collagen or a product called Matrigel™ (BD Biosciences). Collagen is gelled by taking an acidic solution of soluble collagen and neutralizing it; Matrigel is gelled by raising the temperature of a solution thereof from around 10 °C to 37 °C. The gelation process of collagen and Matrigel is slow enough to allow mixing in and encapsulating of cells at a pH and temperature close to physiological conditions. Matrigel is produced from mouse sarcomas and contains a cocktail of growth factors, sugars and undefined proteins, including enzymes. From a cellular perspective, Matrigel can thus be considered as being incredibly ‘information’ rich as it contains a complex mixture of cell-signalling and enzymatic molecules, which is advantageous in that the matrix can be remodelled like ECM and the cells receive cues similar to *in vivo*. However, the complex and ill-defined mixture of components in Matrigel, the batch-to-batch viability, and the lack of physical and chemical control, provide a strong need for other, more defined approaches. This is especially true if high-throughput screening (HTS) assays are required where variations in the substrate could easily overshadow any changes in cell behavior.

The most appealing alternative strategy, that has been pioneered by Hubbell, Lutolf and Anseth, is based on a totally bio-inert synthetic polymer hydrogel substrate decorated with the appropriate signalling molecules providing complete control of the chemistry and mechanical properties.<sup>4-6</sup> HTS 3D assays then become feasible as the first substrate will be identical to the last, irrespective of the batch, and at the same time giving the scientist control over the density and presentation of cell-signalling molecules (at least until the cells begin to produce their own). The natural choice of polymer in these pioneering studies was polyethylene glycol (PEG) – the historical ‘gold standard’ in biologically inert, non-toxic polymers, although PEG is not without its limitations.<sup>7</sup>

A class of polymers that is recently gaining increasing interest as alternatives to PEG are the poly(2-oxazoline)s (full name poly(2-alkyl-2-oxazoline)s, abbreviated PAOx\*).<sup>7,8</sup> PAOx were first reported in the 1960s, but have been largely overlooked for biomedical applications, especially when compared to the more popular, and abundantly available, PEG. When materials were first being selected for use as protein conjugates, PEG was chosen over PAOx as at the time it was more readily available in high purity and the process of “PEGylation” was born.<sup>9</sup> Ever after the FDA approval of PEGylated therapeutics it has been the polymer of choice for other biomedical applications, including 3D hydrogels scaffolds for cell culture.

\* A universal abbreviation has yet to be decided on and poly(2-oxazoline)s are currently being referred to as PAOx, POx, POXA, POz, and POZO while the older literature also uses the term poly(acyl ethylene imine)s.

Unlike PEG, PAOx does not refer to just a single polymer structure but to a class of polymers with a common pseudo-polypeptide backbone and side chain 'R' group. This R group is most commonly a methyl or ethyl group as these polymers are readily soluble in water but it can also contain reactive functionalities in high fidelity. The ability to easily combine monomers with different R and end groups has led to a diverse range of PAOx structures available (Figure 1) with properties ranging from super hydrophilic to amphiphilic, and from inert to reactive leading to a breadth of applications in protein conjugates, micelles, nanoparticles and hydrogels.<sup>8</sup> This large synthetic versatility of PAOx with regard to polymer architecture, functionalities and physiochemical properties is in stark contrast to PEG that is limited to linear and star-shaped polymers bearing only chain-end functionalities. Furthermore, the side-chain is a highly important function of PAOx not found in PEG as it allows not just tuning of the physicochemical properties but also for the incorporation of higher number of bioactive moieties including cell-interacting peptides.

The excellent potential for PAOx as a biomaterial has recently been discussed in several review articles, and this is rapidly gaining attention.<sup>10-12</sup> This increasing interest is reflected in the number of publications from 1966 to 2012 dealing with PAOx (Figure 2) showing a rapid growth in total number of PAOx publications and a blossoming interest in PAOx for the life sciences, especially in the last decade. Besides the synthetic versatility of PAOx as discussed above, the exceptional protein resistance and superior stability of PAOx compared with PEG are other key factors that explain the growing interest in PAOx.<sup>13</sup> Similar to PEG, the protein resistance of PAOx originates from efficient hydration of the polymer chains giving them a 'water-like' structure that excludes protein interactions and gives the polymer 'stealth' properties to proteins and consequently cells. The resistance to other bio-molecules has been successfully exploited in DNA chips<sup>14</sup> and carbohydrate microarrays<sup>15</sup> where background noise due to non-specific binding would render the devices unusable. This combination of bio-stealth behavior and ability to be decorated with functional moieties makes PAOx an interesting candidate for 3D cell culture substrates.

#### *Poly(2-oxazoline) Hydrogels for Controlled Fibroblast Adhesion*

There are many reported strategies for making PAOx hydrogels, yet all of them employ conditions harmful to proteins and cells, such as toxic crosslinkers, organic solvents or high temperatures.<sup>17</sup> Our first attempts of preparing PAOx hydrogels were based on a copolymer with ethyl and 9-decenyl side chains, which turned out to be insoluble in water, so hydrogels had to be prepared in ethanol followed by solvent exchange.<sup>18</sup> Now, we have recently demonstrated a method to synthesize PAOx based hydrogels using mild aqueous curing conditions with the ability to include short peptides covalently bound to the polymer that overcomes this limitation.<sup>19</sup> The approach uses a simple aqueous solution of precursor materials consisting of a water soluble PAOx copolymer with methyl, which is more water-soluble than the ethyl used previously) and 9-decenyl side chains

(pendant alkene groups), a dithiol crosslinker (in this study we used dithiothreitol), thiol-containing peptides (exploiting the thiol in the cysteine residue), and a low percentage of a radical photoinitiator. The solution is then exposed to low flux 365 nm light for several minutes to cure the polymer solution (schematic shown in Figure 3) into a highly transparent crosslinked hydrogel disc that exhibits low autofluorescence making it ideal for high contrast imaging and potential antibody based detection of biomarkers in fixed cells. In this published work, the peptides were first attached to the PAOx followed by crosslinking in a two-step procedure, but recent unpublished experiments have shown that the process works equally well as a one-pot process where the peptide incorporation and crosslinking occur simultaneously. Fibroblast cells were seeded onto these hydrogel discs and were found to be completely non-adherent to the control PAOx hydrogels demonstrating their stealth behavior. When cells were seeded on hydrogel discs containing the well-known cell binding RGD peptide motif common to fibronectin, laminin and collagen, the cells attached and proliferated covering the entire top surface of the hydrogels. This is certainly not the first time that cell integrin binding peptide sequences have been incorporated into hydrogels and cells grown on top, but it was the first instance of it being done with a PAOx hydrogel, thereby providing the first proof-of-principle of PAOx hydrogel scaffolds for cell culture. For further proof of principle we encapsulated the fibroblasts within the hydrogel matrix to demonstrate the possibility of preparing 3D culture. To do this, the cells were mixed with the hydrogel precursor solution and irradiated with light to initiate the curing process. A live/dead assay showed that the cells were largely unaffected by the curing process and were viable within the 3D hydrogel.

The use of light to cure hydrogels has many advantages and indeed it is a commonly employed technique in dentistry as well as for making cell-encapsulated scaffolds for tissue engineering. The thiol-ene reaction used to cure the PAOx hydrogels involves abstraction of a proton from the thiol which subsequently reacts with a carbon-carbon double bond.<sup>20</sup> The reaction can be used under aqueous conditions, is relatively insensitive to oxygen, except in some instances,<sup>21</sup> and works well with the thiol of cysteine meaning that almost any peptide can be incorporated as long as it contains a non-oxidized cysteine residue. The curing reaction can be ‘switched-on’ at will and proceed with predictable kinetics<sup>18</sup> using the light source, which is a distinct advantage over ECM protein hydrogels where the gelation process is difficult to control and adds pressure on the user, especially when trying to simultaneously handle cells in multiple parallel experiments. Light curing also opens up possibilities of creating specific hydrogels patterns and gradients in 2D using photomasks<sup>22</sup> or even in 3D using 2-photon initiation methods<sup>23</sup> and 3D printers.<sup>24</sup> The latter of these, using printers to make 3D shapes from hydrogels is an area of great interest as cells can be incorporated during the printing process and is a method that could be compatible with PAOx via extrusion of the soluble polymer followed by light curing. The use of light, however, is not without risk, especially when used in the presence of cells. This is often overlooked by tissue engineers who use UV light to encapsulate cells in polymer matrices with the goal to create as much new ECM as possible and some collateral cell

damage may not be harmful. For cancer research in 3D, however, the possible DNA dimerization as a result of UV exposure would need to be monitored carefully to ensure that any changes in cell behavior are not due to the curing conditions themselves. We used extremely low power UV irradiation in our study, but, nevertheless, caution is required to avoid unwanted effects on the cells. Strategies to overcome this potential limitation include seeding the cells on top of the post-cured hydrogel and have them migrate in, or to use different curing chemistry. Fortunately, the versatility of PAOx means developing the new chemistry needed is within reach.

#### *Future application of PAOx hydrogels – Cancer research*

As mentioned, we have recently reported the successful attachment and proliferation of fibroblast cells seeded onto RGD functionalized PAOx hydrogels.<sup>19</sup> To assess the utility of PAOx hydrogels for cancer research applications we have extended these results to include both normal (MCF-10A; Figure 4; data not published earlier) and malignant (MCF-7, MDA-MB-231; unpublished data not shown) breast epithelial cell lines. **The hydrogels and conditions were the same as those used for the experiments with fibroblasts as previously reported.**<sup>19</sup> As shown in Figure 4, the highly adherent MCF-10A cell line failed to attach to non-functionalized PAOx hydrogels (left panel) further confirming the protein resistance of the base polymer hydrogel. In contrast, RGD-functionalized hydrogels provided an excellent substrate for cell attachment supporting the proliferation of cells over 14 days (middle and right panels). Importantly, cells grown on RGD-functionalized PAOx hydrogels grew as epithelial colonies with characteristic cortical F-actin organization (Figure 4, middle and right panels), thus maintaining their epithelial phenotype during culture. In addition to monitoring cell attachment, motility and proliferation in 2D, the versatile nature of PAOx hydrogels will allow for the straight-forward inclusion of protease degradable crosslinks. This will open the door to studies of cellular invasion processes as cells secreting specific proteases would degrade the crosslinks, invade and migrate downwards into the PAOx hydrogel forming a 3D invasion model. This may also circumvent the need to encapsulate cells within the hydrogel to study cell invasion. Whether tumor cells are seeded directly on top or encapsulated within hydrogels, PAOx has great potential as an ideal cellular support for 3D studies of cancer cell behavior.

#### *Outlook*

The relatively recent recognition of the fact that studies performed in 3D scaffolds more accurately recapitulate the complex cell-cell and cell-matrix interactions occurring *in vivo* than 2D cell culture, has led to a rapid increase in the number and types of both natural and synthetically derived matrices for 3D culture models.<sup>25</sup> Cancer researchers have been quick to adopt these 3D models into their pre-clinical repertoire to provide more physiologically relevant conditions for the study of tumor

cell biology. These 3D models hold the potential to enable more accurate insights into the response of tumor cells to chemotherapeutic agents, discovery of clinically relevant biomarkers and for identifying molecular and cellular mechanisms underpinning disease progression and metastasis. With the rapidly increasing use of high-throughput functional genomic and drug screening technologies, appropriate, defined and reproducible 3D models are becoming increasingly important for inclusion into these assays to generate more reliable and physiologically relevant “hits” or candidates for further development. Whether 3D models are used in the primary screening assay or for secondary validation studies, the incorporation of these models into HTS studies will undoubtedly increase, and along with it, more robust therapeutic targets will be identified. The potential of PAOx to be functionalized or encapsulated with biological peptides and growth factors, siRNA molecules and therapeutic compounds, at least conceptually, may pave the way for more sophisticated 3D cell culture systems to assess cancer cell biology.

Undoubtedly a number of technical challenges remain before 3D models are routinely used in robust HTS systems,<sup>26,27</sup> but the versatility of synthetic polymers such as PAOx may help to accelerate their use in these systems. More likely than not, any successful approach using PAOx (or any other synthetic polymer) will not just involve the pure functionalized synthetic polymer, but rather hybrid scaffolds of PAOx in combination with recombinant protein-based scaffolds. This makes it a potentially cost-effective approach to prepare 3D structures, which could be conceived to contain spatial segmentation of signalling molecules including morphogens or patterning of structural features to allow for tissue-like hierarchical cellular organization, including vascular channels. High quality PAOx can be synthesized in gram to kilogram quantities relatively cheaply and the chemistry is constantly being improved, including extension of the number of functional groups that can be incorporated. The commercial availability of new PAOx polymers is ever increasing and companies such as Serina Therapeutics<sup>28</sup> (drug delivery) and GATT Technologies<sup>29</sup> (tissue sealants) are paving the way for the inevitable wide-spread use of this highly potent class of polymers.

## Acknowledgments

TD and BH are grateful for funding from the Cells and Tissues Domain at QUT and to the Australian Research Council (LP110201004). RH thanks Ghent University for funding via the Concerted Research Actions scheme.

## References

1. Nelson CM, Inman JL, Bissell MJ, Three-dimensional lithographically defined organotypic tissue arrays for quantitative analysis of morphogenesis and neoplastic progression. *Nat Protoc* 2008; 3, 674-678

2. Abbott A, Cyranoski D. Biology's new dimension. *Nature* 2003; 424, 870-872
3. Dalton PD, Vaquette C, Farrugia BL, Dargaville TR, Brown TD, Hutmacher DW. Electrospinning and additive manufacturing: converging technologies. *Biomater Sci* 2013; 1, 171-185
4. Lutolf MP, Lauer-Fields JL, Schmoekel HG, Metters AT, Weber FE, Fields GB, Hubbell JA. Synthetic matrix metalloproteinase-sensitive hydrogels for the conduction of tissue regeneration: Engineering cell-invasion characteristics. *Proc Natl Acad Sci USA* 2003; 100, 5413–5418
5. Ehrbar M, Rizzi SC, Schoenmakers RG, San Miguel B, Hubbell JA, Weber FE, Lutolf MP. Biomolecular hydrogels formed and degraded via site-specific enzymatic reactions. *Biomacromolecules* 2007; 8, 3000-3007
6. DeForest CA, Anseth KS. Cytocompatible click-based hydrogels with dynamically-tunable properties through orthogonal photoconjugation and photocleavage reactions. *Nat Chem* 2011; 3, 925-931
7. Knop K, Hoogenboom R, Fischer D, Schubert US. Poly(ethylene glycol) in drug delivery: Pros and cons as well as potential alternatives. *Angew Chem Int Ed* 2010; 49, 6288-6308
8. Hoogenboom, R. Poly(2-oxazoline)s: a polymer class with numerous potential applications. *Angew Chem Int Ed* 2009; 48, 7978-7994
9. Davis FF, The origin of peganology. *Adv Drug Deliv Rev* 2002; 54, 457–458
10. Sedlacek O, Monnery BD, Filippov SK, Hoogenboom R, Hruby M. Poly(2-oxazoline)s – are they more advantageous for biomedical applications than other polymers? *Macromol Rapid Commun* 2012; 33, 1648-1662
11. Luxenhofer R, Han Y, Schulz A, Tong J, He Z, Kabanov AV, Jordan R. Poly(2-oxazoline)s as polymer therapeutics. *Macromol Rapid Commun* 2012; 33, 1613-1631
12. Barz M, Luxenhofer R, Zentel R, Vicent MJ. Overcoming the PEG-addiction: well-defined alternatives to PEG, from structure–property relationships to better defined therapeutics. *Polym Chem* 2011; 2, 1900
13. Pidhatika B, Rodenstein M, Chen Y, Rakhmatullina E, Mühlebach A, Acikgöz C, Textor M, Konradi R. Comparative stability studies of poly(2-methyl-2-oxazoline) and poly(ethylene glycol) brush coatings. *Biointerphases* 2012; 7, 1-4
14. Hartlieb M, Pretzel D, Kempe K, Fritzsche C, Paulus, RM, Gottschaldt M, Schubert US. Cationic poly(2-oxazoline) hydrogels for reversible DNA binding. *Soft Matter* 2013; 9, 4693-4704

15. Wang H, Li L, Tong Q, Yan M. Evaluation of photochemically immobilized poly(2-ethyl-2-oxazoline) thin films as protein-resistant surfaces. *ACS Appl Mater Interfaces* 2011; 3, 3463–3471
16. SciFinder search using terms ‘poly’ AND ‘oxazoline’ refined to journal and review articles; and refined to ‘biology’ or ‘biomaterials’
17. Kelly AM, Wiesbrock F. Strategies for the Synthesis of Poly(2-Oxazoline)-Based Hydrogels. *Macromol Rapid Commun* 2012; 33, 1632–1647
18. Dargaville TR, Forster R, Farrugia BL, Kempe K, Voorhaar L, Schubert US, Hoogenboom R. Poly(2-oxazoline) hydrogel monoliths via thiol-ene coupling. *Macromol Rapid Commun* 2012; 33, 1695-1700
19. Farrugia BL, Kempe K, Schubert US, Hoogenboom R, Dargaville TR. Poly(2-oxazoline) hydrogels for controlled fibroblast attachment. *Biomacromolecules* 2013; 14, 2724–2732
20. Lowe A. Thiol-ene “click” reactions and recent applications in polymer and materials synthesis. *Polym Chem* 2010; 1, 17–36
21. Keddie DJ, Grande JB, Gonzaga F, Brook MA, Dargaville TR. Amphiphilic silicone architectures via anaerobic thiol-ene chemistry. *Org Lett* 2011; 13, 6006–6009
22. Beria L, Gevrek TN, Erdog A, Sanyal R, Pasini D, Sanyal A. ‘Clickable’ hydrogels for all: facile fabrication and functionalization. *Biomater Sci* 2013; 2, 67-75
23. Torgensen J, Qin X, Li Z, Ovsianikov A, Liska R, Stampfl J. Hydrogels for two-photon polymerization: a toolbox for mimicking the extracellular matrix. *Adv Funct Mat* 2013; 23, 4542-4554
24. Bakarich SE, in het Panhuis M, Beirne S, Wallace GG, Spinks GM. Extrusion printing of ionic-covalent entanglement hydrogels with high toughness. *J Mater Chem B* 2013; 1, 4939-4946
25. Hutmacher DW, Loessner D, Rizzi S, Kaplan DL, Mooney DJ, Clements JA. Can tissue engineering concepts advance tumor biology research? *Trends Biotechnol* 2010; 28, 1250
26. Friedrich J, Ebner R, Kunz-Schughart LA. Experimental anti-tumor therapy in 3-D: spheroids—old hat or new challenge? *Int J Radiat Biol* 2007; 83, 84907
27. LaBarbera DV, Reid BG, Yoo BH. The multicellular tumor spheroid model for high-throughput cancer drug discovery. *Expert Opin Drug Discov* 2012; 7(9), 819-830
28. [www.serinatherapeutics.com](http://www.serinatherapeutics.com) (accessed 30 Sept 2013)
29. [www.gatt-tech.com](http://www.gatt-tech.com) (accessed 30 Sept 2013)



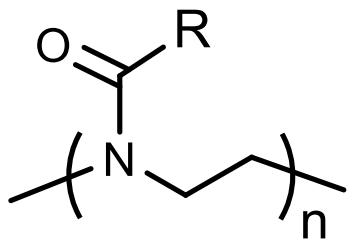
## Figure Captions

**Figure 1:** General structure of poly(2-oxazoline)s (top left) and a selection of easily accessible functional PAOx structures ranging from linear with variable chain end or side chain functionalities to stars highlighting the structural versatility of this class of polymers.

**Figure 2:** The popularity of PAOx highlighted by the cumulative number of publications in all fields (top line) and in biology and biomaterials (bottom line) from 1966 to 2012. *Source: SciFinder*<sup>16</sup>

**Figure 3:** Synthesis of functional PAOx hydrogels. Schematic showing the modular approach of attaching peptides to the polymer side-chains and crosslinking.

**Figure 4:** Confocal microscopy images of MCF-10A cells seeded onto PAOx hydrogels with no RGD (left panel) and functionalized with RGD (middle and right panel). Cells were grown in regular growth media for 14 days before being stained with phalloidin and DAPI. Scale bars shown.



linear asymmetric



linear asymmetric (bifunctional)



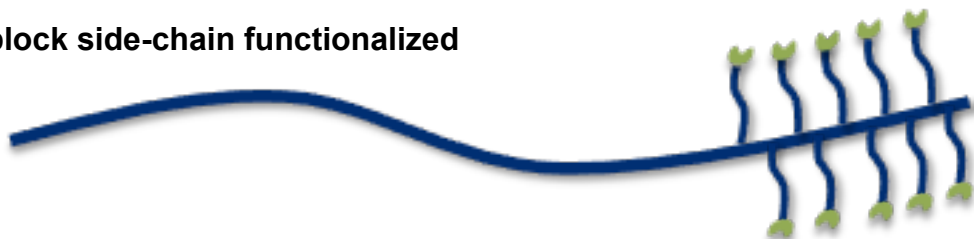
random side-chain functionalized



gradient side-chain functionalized



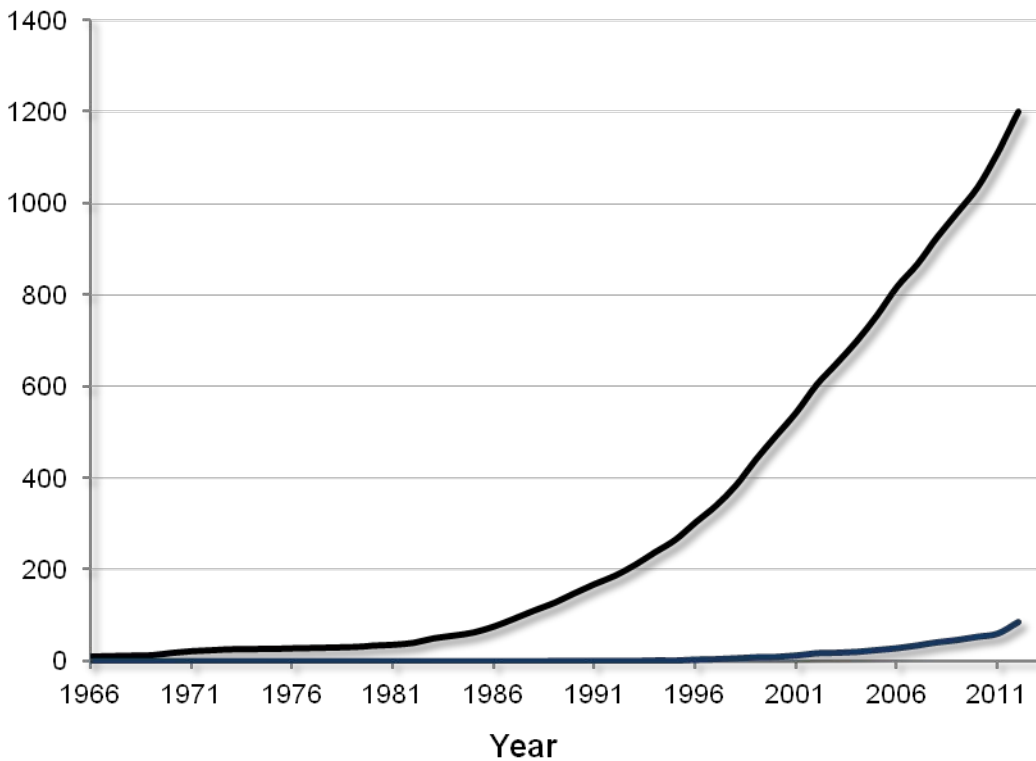
block side-chain functionalized



stars



Cumulative Publications



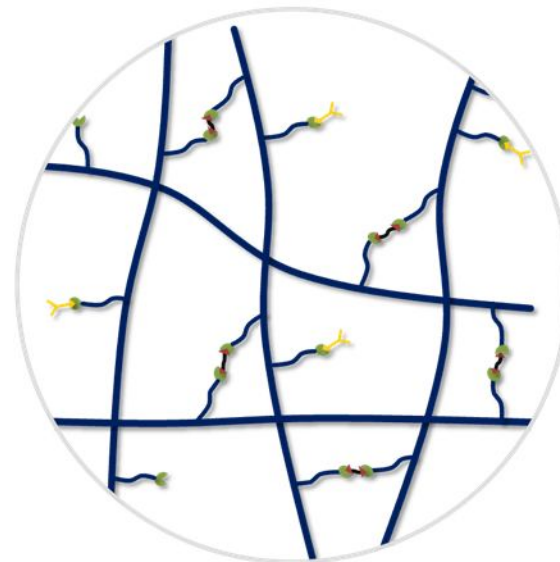


functional POx

peptide



dithiol crosslinker



hydrogel network

